INFECTION AND IMMUNITY, June 1990, p. 1843–1847
0019-9567/90/061843-05$02.00/0

Vol. 58, No. 6

Erythrocyte Receptor (CD2)-Bearing T Lymphocytes Are Affected by Diet in Experimental Pulmonary Tuberculosis

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Received 6 February 1990/Accepted 2 April 1990

Outbred (Hartley) and inbred (strain 2 and 13) guinea pigs were vaccinated with *Mycobacterium bovis* BCG and placed on isocaloric, purified diets containing either 10 or 30% ovalbumin or on commercial guinea pig chow. Six weeks later, the animals were challenged by the respiratory route with virulent *M. tuberculosis* H37Rv. At intervals postchallenge, groups were tuberculin tested and sacrificed. Thymus-dependent (T) lymphocytes were enumerated either by rosette formation with rabbit erythrocytes or by the indirect immunofluorescence assay (IFA) with a guinea pig pan-T-cell monoclonal antibody, 8BE6. Protein-deficient guinea pigs of all three strains had significantly fewer erythrocyte rosette-forming ($CD2^+$) T cells in the peripheral blood, and malnourished strain 2 and Hartley guinea pigs exhibited reduced levels of $CD2^+$ T cells in the thymus. In contrast, animals of all three strains fed the low-protein diet harbored more $CD2^+$ T cells in the bronchotracheal lymph nodes than did their control-fed counterparts. A larger proportion of lymphocytes from the blood and lymph nodes of all three strains were IFA positive than formed erythrocyte rosettes regardless of diet treatment. Diet had no effect on IFA-positive lymphocytes in those organs. Protein deficiency is associated with significant alterations in the number and/or distribution of T lymphocytes expressing functional CD2-receptors in BCG-vaccinated animals exposed to virulent mycobacteria by the pulmonary route. These alterations may contribute to the reduction in BCG vaccine efficacy observed in this model.

The immune mechanisms of resistance against infection with *Mycobacterium tuberculosis* depend upon a complex interplay of host cells, with T lymphocytes occupying the pivotal role (7, 10). The T-cell population engaged in host defense is comprised of the helper-inducer (CD4) and suppressor-cytotoxic (CD8) subsets as well as T cells expressing receptors for the Fc portion of immunoglobulin (8, 14). Resistance may be mediated directly through the cytolytic actions of CD8 cells against infected macrophages or indirectly by the activation of monocytes or macrophages by lymphokine products of antigen-sensitized T lymphocytes (13).

Prior to the introduction of monoclonal antibodies, human T cells were recognized by their ability to form rosettes spontaneously with sheep erythrocytes (22). The T-cell surface molecule responsible for this fortuitous interaction is the 50-kilodalton glycoprotein, CD2 (E receptor, T11) (26). The physiologic relevance of the CD2 receptor may lie in its role in T-cell adhesion and activation processes, including lymphokine (e.g., interleukin-2) production and clonal expansion (4, 12). The expression of CD2 can be up-regulated by the activation of lymphocytes with interleukin-1, alloantigens, or mitogens (27). The modulation of CD2 expression may be a measure of the maturity or activation potential of T cells. Recently, a relationship between immunological unresponsiveness in lepromatous leprosy patients and down-regulation of E receptors on peripheral T lymphocytes was investigated, with conflicting results (23, 24, 31).

The T-cell arm of the immune response is predictably and profoundly compromised by dietary deficiencies (15). Malnourished patients consistently present with a significant reduction in CD4- and E receptor-bearing T cells (2, 3). Using a well-established guinea pig model of experimental pulmonary tuberculosis, we have demonstrated that chronic, moderate protein deficiency severely compromises purified protein derivative-induced T-cell functions and reduces the efficacy of *Mycobacterium bovis* BCG vaccination (6, 16, 18–20). These diet-related alterations may be mediated by changes in the number, distribution, or development of T lymphocytes in protein-deficient guinea pigs (17), suggesting that these animals may fail to mobilize and expand the appropriate clones of antigen-reactive T cells.

In this study we have examined the impact of dietary protein on the number and distribution of T lymphocytes in three strains of guinea pigs vaccinated with BCG and infected by the respiratory route with virulent *M. tuberculosis*. The rabbit erythrocyte rosette assay was used to quantify T lymphocytes expressing functional E receptors (CD2) (28). A pan-T-cell monoclonal antibody for guinea pigs (8BE6) provided an independent phenotypic measure of T-cell numbers (5). Protein malnutrition exerted a significant effect on E receptor (CD2)-bearing T cells in both inbred (strain 2 and 13) and outbred (Hartley) guinea pigs infected with *M. tuberculosis*.

(This study was previously presented in part [R. A. Bartow, C. L. Mintzer, and D. N. McMurray, Fed. Proc. **45**:707, 1986].)

MATERIALS AND METHODS

Experimental animals. Specific-pathogen-free, outbred albino guinea pigs (Hartley-COBS, Crl:(HA)Br; Charles River Breeding Laboratories, Inc., Wilmington, Mass.) and inbred strain 2 and strain 13 guinea pigs (Veterinary Resources Division, University of Texas System Science Park, Bastrop), weighing initially between 150 and 200 g, were obtained for this study. The animals were housed individually in polycarbonate cages with stainless steel grid floors and feeders and were allowed food and water ad libitum. Each animal was randomly assigned to an experimental diet. Body weights were recorded weekly throughout the experiment.

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Experimental diets. Purified experimental diets were obtained from a commercial supplier (Dyets Inc., Bethlehem, Pa.) and formulated to our specifications by inversely varying the proportions of cornstarch and ovalbumin to obtain the desired protein content. The exact diet composition was published previously (21). The control diet contained 30% ovalbumin as the sole protein source. The low-protein diet was isocaloric and identical to the control diet in every nutrient except protein (10% ovalbumin). The third diet used was commercial guinea pig chow (Ralston Purina, St. Louis, Mo.). Prior to the initiation of experimental diets, animals were weaned from commercial chow by being fed a 50:50 mixture of ground chow and powdered control diet for 2 weeks.

BCG vaccination. All guinea pigs received a subcutaneous injection of 0.1 ml of *M. bovis* BCG (Copenhagen 1331; Statens Seruminstitut, Copenhagen, Denmark) in the left inguinal region. The lyophilized BCG vaccine was reconstituted with sterile physiological saline to deliver approximately 10^3 viable organisms per animal.

Respiratory infection. *M. tuberculosis* H37Rv (ATCC 27294) was obtained from the American Type Culture Collection, Rockville, Md., and stored as single-cell suspensions at −70°C (11). The challenge inoculum was thawed and diluted just prior to infection. All animals were infected via the respiratory route by use of an aerosol chamber described previously (30). The infecting inoculum of viable *M. tuberculosis* H37Rv introduced into the nebulizer was empirically adjusted to result in the inhalation and retention of 5 to 10 viable organisms per animal. The infection was performed in a biohazard facility designed for use with class 3 human pathogens. Exposure of groups of guinea pigs, selected randomly from the diet groups, resulted in uniform, reproducible infection of all animals with mycobacteria.

Necropsy procedure. Guinea pigs were killed by the intramuscular injection of 1 to 3 ml of sodium pentobarbital (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) at weekly intervals following respiratory infection. A blood sample was obtained immediately via cardiac puncture. Peripheral blood lymphocytes were isolated by density gradient centrifugation in lymphocyte separation medium (Organon Teknika Corp., Durham, N.C.). Lymphocytes at the interface were removed and washed three times in phosphate-buffered saline (PBS) supplemented with 2% fetal bovine serum. Single-cell suspensions of thymus and bronchotracheal lymph nodes were obtained by gently homogenizing the tissue in tissue culture medium (RPMI 1640) in a sterile Ten Broeck homogenizer. Lymphocyte suspensions were assessed for viability by trypan blue exclusion and adjusted to 2×10^6 cells per ml.

T-cell rosette (CD2) assay. The number of $CD2^+$ T cells in lymphocyte populations isolated from blood and lymphoid tissues was quantified by the rabbit erythrocyte rosette assay (28). Rabbit blood was stored in Alsever solution at 4°C. The whole blood was centrifuged and washed two times in PBS–2% fetal bovine serum prior to use. Erythrocytes were counted in a hemacytometer and adjusted to 100×10^6 cells per ml. Rosettes were prepared by incubating 200 μl of lymphoid suspension and 200 μl of rabbit erythrocytes in a borosilicate glass culture tube (10 by 75 mm) at 37°C for 30 min. The cells were centrifuged for 5 min at $200 \times g$ at 4°C and kept overnight at 4°C. The pellet was resuspended by gently rolling the culture tube. Two drops of 0.4% trypan blue were added to the tube, and rosettes were counted in a hemacytometer. A rosette ($CD2^+$ T cell) was defined as a lymphocyte with three or more adherent rabbit erythrocytes.

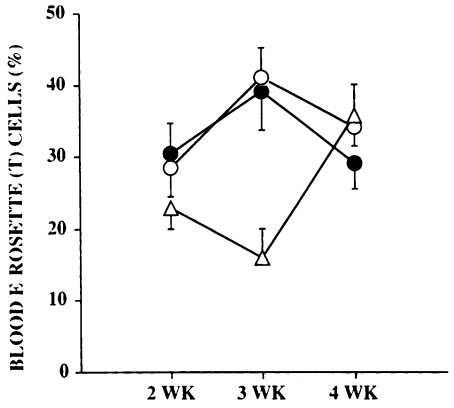

FIG. 1. Proportion of erythrocyte rosette (E rosette)-forming ($CD2^+$) T lymphocytes in the blood of BCG-vaccinated Hartley strain guinea pigs fed commercial chow (○) or purified control (●) or low-protein (△) diets and sacrificed following pulmonary infection with virulent *M. tuberculosis* H37Rv. Each point represents the means ± standard errors of the means for four to six animals.

Rosettes were expressed as a percentage of total viable lymphocytes.

Indirect immunofluorescence assay. Total T cells were quantified in an indirect immunofluorescence assay with the 8BE6 pan-T-cell monoclonal antibody for guinea pigs generously provided by Ethan Shevach (National Institutes of Health, Bethesda, Md.) (5). Lymphocytes were washed three times in PBS–5% fetal bovine serum supplemented with 0.1% sodium azide. Lymphocyte suspensions (10^7 cells per ml) were incubated with an equal volume of 8BE6 culture supernatant for 45 min at 4°C. Cells were washed two times in 3 to 5 ml of cold supplemented PBS and suspended in 100 μl of PBS. An equal volume of commercial anti-mouse gamma globulin conjugated with fluorescein isothiocyanate (Organon Teknika) diluted 1:300 in PBS was added and incubated for 45 min at 4°C. Cells were washed once in supplemented PBS and once in mounting fluid (PBS with 3% bovine serum albumin, 0.1% sodium azide, 10% glycerol, and 100 μg of *o*-phenylenediamine per ml). The pellet was suspended in mounting fluid.

The number of fluorescent lymphocytes was counted under oil immersion with a Zeiss epifluorescence microscope. The total lymphocyte count was obtained by enumerating the same area under bright-field illumination, and the results were expressed as a percentage. Control slides were devised by replacing 100 μl of primary antibody (8BE6) with 100 μl of PBS.

Analysis of variance was utilized to test the effects of dietary protein content on the dependent variables measured. When significant treatment effects were indicated, differences between means were assessed by the new multiple-range test of Duncan (29). A 95% confidence level was set for all tests.

RESULTS

Figure 1 illustrates the influence of diet on the proportions of erythrocyte rosette-forming ($CD2^+$) T lymphocytes in the peripheral blood of outbred Hartley guinea pigs at weekly intervals postchallenge with virulent *M. tuberculosis* H37Rv. Protein deficiency was accompanied by a dramatic reduction ($P < 0.01$) in the proportion of circulating $CD2^+$ T

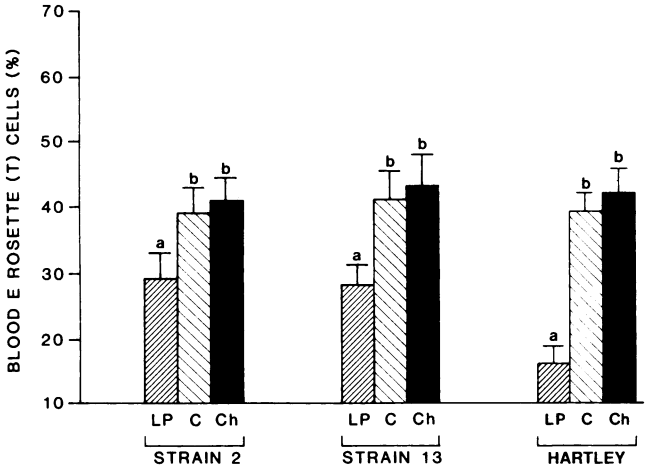

FIG. 2. Circulating $CD2^+$ T cells in three strains of BCG-vaccinated guinea pigs consuming low-protein (LP) or control (C) purified diets or commercial chow (Ch) and challenged by the respiratory route 2 to 3 weeks earlier with virulent *M. tuberculosis* H37Rv. The data represent the means ± standard errors of the means for four to six animals per treatment; means denoted by the same letter within each strain are not significantly different ($P > 0.05$). E, Erythrocyte.

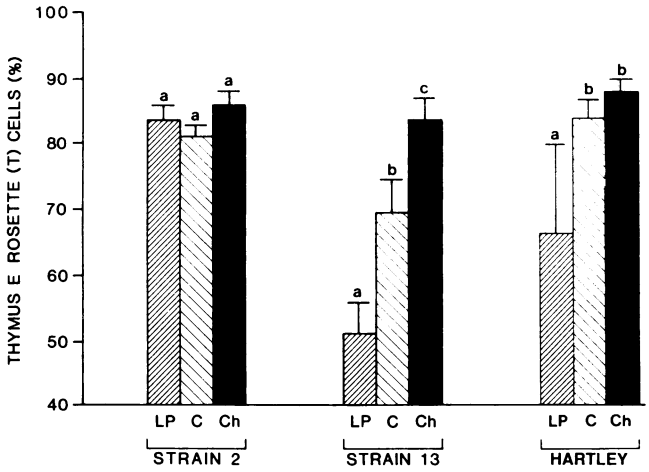

FIG. 3. Thymic $CD2^+$ T cells in three strains of BCG-vaccinated guinea pigs consuming low-protein (LP) or control (C) purified diets or commercial chow (Ch) and challenged 2 to 3 weeks earlier with virulent *M. tuberculosis* H37Rv. The data represent the means ± standard errors of the means for four to six animals per treatment; means denoted by the same letter within each strain are not significantly different ($P > 0.05$). E, Erythrocyte.

cells at 3 weeks. This depression was temporary, with the levels recovering to normal by 4 weeks. This temporal dietary effect was observed repeatedly and varied between 2 and 3 weeks in other experiments. At 3 weeks, the protein-malnourished animals had less than half of the $CD2^+$ T cells observed in normally nourished (control or chow) guinea pigs.

Dietary protein deficiency also affected the levels of $CD2^+$ T cells in the peripheral blood of inbred strain 2 and strain 13 guinea pigs 2 to 3 weeks following pulmonary infection with virulent mycobacteria (Fig. 2). Data from Hartley animals are included for comparison. The decrease in the levels of $CD2^+$ T cells, while statistically significant ($P < 0.05$), was not a dramatic in inbred, protein-deprived guinea pigs as in their outbred counterparts. Strain 2 and 13 animals fed the low-protein diet had nearly twice as many $CD2^+$ T cells in their circulation as did Hartley guinea pigs fed the same deficient diet but were still $CD2^+$ T-cell deficient as compared with normally nourished animals.

As expected, 85 to 90% of thymic lymphocytes recovered from normal, chow-fed guinea pigs of all three strains expressed functional E receptors (Fig. 3). The effect of dietary treatment on the proportions of $CD2^+$ T cells in the thymus varied between guinea pig strains. Protein deficiency was accompanied by a marked diminution ($P < 0.05$) in thymic levels of $CD2^+$ T cells in outbred Hartley and inbred strain 13 animals but had no measurable effect in the strain 2 guinea pigs. Strain 13 animals fed the purified control diet exhibited a modest but statistically significant reduction in erythrocyte rosette-forming T cells in the thymus which was not observed in the guinea pigs of the other two strains fed the control diet.

To determine whether the diet-induced alterations in the populations of $CD2^+$ T cells reflected a reduction in total, mature T cells in the tissues, we assessed an independent phenotypic T-cell marker. Table 1 compares the proportions of T lymphocytes binding the pan-T-cell monoclonal antibody (8BE6) or forming erythrocyte rosettes in the blood or bronchotracheal lymph nodes of protein-deficient or control strain 2 guinea pigs. Animals were sacrificed 2 and 3 weeks following pulmonary challenge with virulent *M. tuberculosis* H37Rv.

In the blood, protein deprivation resulted in a temporary but significant ($P < 0.05$) reduction in $CD2^+$ T cells at 2 weeks which rose to control values by 3 weeks postchallenge. This dietary effect was not observed with the IFA for $8BE6^+$ T cells, which were present in significantly higher proportions than $CD2^+$ T cells in the circulation of protein-deficient guinea pigs at 2 weeks. There were no statistically significant differences between $8BE6^+$ and $CD2^+$ T cells in the blood of normally nourished animals at either sacrifice interval.

Lymph node cells from both diet groups consistently contained significantly ($P < 0.05$) more $8BE6^+$ T cells than erythrocyte rosette-forming T cells at both intervals. In contrast to the temporary decrease in the levels of $CD2^+$ T lymphocytes in the blood of protein-deficient guinea pigs, the bronchotracheal lymph nodes of these same animals contained significantly more $CD2^+$ T cells at both 2 and 3 weeks postchallenge ($P < 0.05$).

TABLE 1. Effect of dietary protein on the proportion of T lymphocytes labeled with a pan-T-cell monoclonal antibody (8BE6) or expressing the E receptor (CD2) in the tissues of tuberculous strain 2 guinea pigs

Diet	Interval post-challenge (wk)	% of total lymphocytes[a] in:			
		Blood		Lymph nodes	
		$8BE6^+$	$CD2^+$	$8BE6^+$	$CD2^+$
Low protein	2	37.1 ± 2.8	24.7 ± 4.1[b]	47.3 ± 4.2	40.0 ± 1.8[c]
	3	34.0 ± 3.0	38.6 ± 3.8[b]	47.4 ± 2.1	39.1 ± 1.9[d]
Control	2	33.5 ± 3.1	32.0 ± 4.0	45.5 ± 2.8	35.8 ± 1.6[c]
	3	40.2 ± 2.0	34.7 ± 3.6	46.0 ± 1.8	34.2 ± 0.8[d]

[a] Means ± standard errors of the means for four to five animals per treatment. Differences between values marked with the same letter are statistically significant ($P < 0.05$).

DISCUSSION

This study documents the effect of chronic, moderate protein malnutrition on the proportion and distribution of T lymphocytes in BCG-vaccinated guinea pigs infected by the respiratory route with virulent *M. tuberculosis*. Protein deficiency has been shown to impede the protection normally afforded by the BCG vaccine against pulmonary challenge in this model (6, 18, 20). The results presented here demonstrate that diet-induced alterations in cellular immunity are accompanied by changes in the percentages of T lymphocytes expressing a functional CD2 receptor.

All three strains of guinea pigs maintained on protein-deficient diets exhibited a significant, albeit transient, reduction in the percentage of circulating $CD2^+$ T lymphocytes. A profound diminution in $CD2^+$ T cells was also observed in the thymus in two of three strains. Conversely, nutritional compromise yielded higher percentages of $CD2^+$ T cells in the bronchotracheal lymph nodes draining the infection site in protein-deficient animals as compared with fully nourished controls. CD2 modulation as observed in protein deficiency was not paralleled in studies of lymphocytes labeled with a guinea pig pan-T-cell monoclonal antibody (5). Diet was not demonstrated to influence 8BE6 receptor expression in any tissue examined. Functional CD2 expression appears to be a sensitive indicator of dietary insult. Protein deficiency may modulate CD2 expression, resulting either in the functional depletion of activated T cells or in the sequestration of $CD2^+$ T cells, rendering them unavailable to participate in the immune response to mycobacteria.

Although CD2 expression on T cells in guinea pigs has not been thoroughly investigated, it appears that the guinea pig antigen responsible for binding to rabbit erythrocytes is analogous to a human T-cell differentiation antigen with a molecular size of 50 to 65 kilodaltons (26). The ability to form rosettes is acquired during thymic maturation, and CD2 expression can be up-regulated on guinea pig splenocytes by exposure to thymic extract (9, 25, 28). The natural ligand of CD2 in the human system is lymphocyte function-associated antigen 3, which allows the CD2 receptor to serve as an adhesion molecule (27). CD2 participates in T-cell activation by affecting signal transduction and interleukin-2 production (4). The alterations in CD2 expression and/or the distribution of $CD2^+$ T cells observed in this study in protein-deficient guinea pigs may result in a loss of functional trafficking capabilities and/or a compromise in interleukin-2 production in response to antigen. We recently reported a decrease in the generation of interleukin-2 by lymphocytes from protein-malnourished animals (19).

The finding of reduced numbers of $CD2^+$ lymphocytes in the thymus in strain 13 and Hartley guinea pigs is suggestive of a compromise in T-lymphocyte differentiation resulting in a departure of immature T cells from the thymus. Chandra has demonstrated an increased null cell population in malnourished children, as well as elevated levels of leukocyte terminal deoxynucleotidyltransferase, an enzyme found almost exclusively in immature T cells (3). Thymic hypoplasia, as well as reduced levels of thymic hormones, has been identified in conjunction with protein malnourishment and may directly affect CD2 expression (15). We are currently investigating the influence of protein deficiency on thymic architecture and circulating thymulin levels in this model.

We recently reported a significant dietary effect on the expression and/or distribution of an independent T-cell surface marker, the receptor for the Fc portion of immunoglobulin, in tuberculous guinea pigs. T lymphocytes expressing a functional Fc receptor for immunoglobulin G (Tγ cells) were augmented in the circulation and bronchotracheal lymph nodes of protein-deprived guinea pigs postchallenge, while the levels of T lymphocytes bearing an immunoglobulin M Fc receptor (Tμ cells) were consistently reduced in the lymph nodes (17). We are presently conducting experiments to ascertain the relationship, if any, between CD2 and Fc receptor expression on T lymphocytes in our model.

CD2 modulation and immune responses have been studied in another mycobacterial disease, leprosy, with conflicting results. Muthukkaruppan et al. (23, 24) demonstrated down-regulation of the CD2 molecule by exposure to *Mycobacterium leprae*. However, Wong et al. (31) recently reported normal percentages of $CD2^+$ T cells in lepromatous leprosy skin lesions and on peripheral T lymphocytes activated by anti-CD2 antibodies. One explanation for this discrepancy may be that the use of actual rosette formation in the former studies, as opposed to monoclonal antibody labeling in the latter, reflects the presence of a functional E receptor. While the mere presence of the CD2 protein may be sufficient for some functions, other roles may require this glycoprotein to be expressed in a specific conformation (e.g., glycosylated or sialylated) or at a certain density (1). Our data, obtained with rosette formation as the endpoint, support the contention that the loss of antimycobacterial resistance which accompanies chronic protein deficiency may be associated with alterations in the expression of CD2 or the distribution of $CD2^+$ T cells.

ACKNOWLEDGMENTS

We thank Carole L. Mintzer for excellent technical assistance and Jane Lantz for secretarial support.

This work was supported by Public Health Service grant AI-15495 from the National Institute of Allergy and Infectious Diseases.

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